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13. ABSTRACT (Maximum 200 Words)

The aromatic hydrocarbon (or dioxin) receptor (AhR) which is a ligand-activated basic helix-loop-helix (bHLH) transcription factor, mediates the toxic responses of dioxin. The research in this proposal is testing the hypothesis that the dioxin receptor (AhR) plays a central role in regulating the mammary epithelial network during the gland development, as well as regulating major events in mammary carcinogenesis. Following on preliminary observation of the dramatic upregulation of this receptor in advanced human breast carcinoma (HBC) cell lines, we proposed to address the question of whether the AhR overexpression alone is sufficient for transforming normal mammary epithelia, and whether it is causally associated transformation, using two genetic approaches. The AhR expression will be blocked in high tumorigenic HBC cell lines by siRNA technology to demonstrate a direct role of the AhR in modifying the progression of metastasis. To directly address the effect of increased expression of AhR, the human AhR cDNA will be stably transfected and over-expressed in a normal mammary epithelia and in non-tumorigenic human breast cell line. The development of metastatic phenotypes in the AhR-transformed lines will be assayed as their ability for anchorage-independent growth in soft agar media and for inducing tumors in nude mouse.

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Introduction

The proposed research studies are based on our novel observation that in three different sets of human breast carcinoma (HBC) cell lines the expression of the Aryl hydrocarbon receptor (AhR) protein was up-regulated in a direct correlation with the progression of tumorigenecity. The overall objective is to determine the role of AhR in human breast carcinogenesis. AhR which is well known for its mediation of the toxic responses to environmental polyhalogenated aromatic hydrocarbons (PAH) such as dioxin (1), is a cytosolic protein and binding of PAH leads to its activation to a nuclear transcription factor and subsequent down-regulation by proteolysis (2,3,4). Molecular cloning and characterization of AhR cDNA has identified it as a member of family of ligand-activated basic helix loop helix (bHLH) transcription factors (5). PAH-activated AhR heterodimerizes with its partner AhR nuclear translocating protein (ARNT) another bHLH transcription factor and induces the expression of a number of genes, including cytochrome P4501A1 (CYP1A1) and CYP1B1 (reviewed in 6 & 7). No endogenous ligand for the AhR has yet been identified, however, its constitutive activation via disturbing cellular adhesion to the extra-cellular matrix (8,9), increasing intracellular $Ca2+$ (10), and disturbing cytoskeleton (11) has provided evidence for physiologically-activated pathways linked to adhesion.

Furthermore, other lines of evidence are gathering to implicate the AhR in normal development and tissue homeostasis. Its long been established that TCDD exposure in animals induces teratogesis, immunosuppression, reproductive defects and tumor promotion, in an AhR-dependent manner. The dioxin-dependent activation of the AhR has also been linked to inhibition of proliferation in mammary and uterine tumor cell lines (reviewed in 12), and enhanced terminal differentiation in keratinocytes and palatal epithelia (13,14). Moreover, the AhR null mice generated by two independent laboratories, although they are normal, they exhibit a spectrum of hepatic and immune system defects (15,16), but are resistant to benzo(a)pyrene-induced skin and liver carcinogenicity (17).

In cultured cells, TCDD shows marked effects on cell cycle progression, where it induced a cell cycle arrest at G1/S check point, an effect that is mediated through the AhR and it involves the induction of cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$. The AhR in absence of dioxin or other ligands, was shown to influence cell cycle progression, cell shape and differentiation (18, 19). This effect on the cell cycle progression relies on a direct protein-protein interaction of AhR with retinoblastoma (Rb) through an LXCXE domain on the AhR (20). Howeverr, studies in MCF-7 showed that AhR associates with Rb only after receptor activation and nuclear translocation (21). Furthermore, this interaction with Rb protein is required for the maximal AhR transcriptional activity (22).

Although TCDD acting through AhR is a potent tumor promoter in mouse skin and in rat liver, it has strong anti-mitogenic effect in estrogen-responsive tissues and exhibits a broad spectrum of anti-estrogenic activities in human breast carcinoma cells (reviewed in 12). The AhR-null mouse or normal mouse treated with TCDD, exhibited impaired development of mammary gland ductal branching (23). Taken together with the anti-mitogenic effect exerted by TCDD on these cells, and the fact that TCDD down-regulates the AhR subsequent to its activation, these findings suggests that AhR is involved in regulating the proliferative stage required for mammary gland development. Preliminary investigations in our laboratory have demonstrated the expression of high levels of AhR protein in human mammary carcinoma cell lines in direct proportion to their degree of tumorigenicity and metastatic potential (24). We hypothesize that the AhR plays a major role in regulating mammary epithelial network during mammary gland development, and its over-expression in human breast carcinoma contributes to the development of metastatic phenotypes.

The goal of this work has been to investigate the mechanisms of AhR involvement in regulating mammary epithelia both during development and tumoigenesis. Our approach during this first year has been to

develop the tools and reagents to establish these goals. Although we proposed a slightly different regimen, in the interest of the time and reagents we found we had to do some adjustment in the working plan. For instance, some of the work which involves examining activation status of the AhR in Sager's cells, has been addressed this year although we proposed to do next year.

Progress-Year 1

Objective 1: *Restoration of the normal mammary development in the AhR-KO mice by transfection of AhR cDNA into their mammary epithelial cells in situ using retroviral expression vectors*

Our initial task was to construct recombinant retroviral expression vectors for both the mouse and human AhR genes. This was to be followed by the production of high titer virus stocks of the respective gene for subsequent experimentations. Although in our Statement of Work we proposed to finish these tasks within six months however, that was over-ambitious goal that significantly under-estimated the time required for these tasks. This delay is in part attributed to the mere difficulty in establishing new technologies, however, most of the delay we encountered was due to problems with the commercial kits we used.

1. Construction of the recombinant retroviral expression vector for mouse AhR:

We used *Pfx* DNA polymerase to amplify full-length mouse AhR cDNA flanked by two restriction sites for CIa-I and Xho-1. The restriction sites were selected as adaptor for the cloning sites in the retrovirus vector pLNCX2 (Retro-X-System provided by Clontech).

2.4kb ➡

Original PCR   gel Purified

Fig1 shows PCR product of mouse AhR cDNA
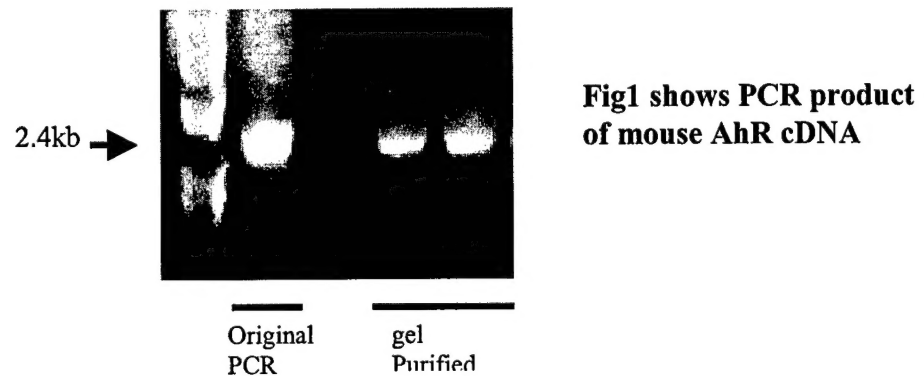
We sub-cloned the PCR products into pCR-BluntII-TOPO cloning vector of Zero Blunt TOPO PCR cloning Kit to test right sequence and restriction sites. The mouse AhR insert was then cut out from this TOPO cloning vector with CIa-I and Xho-1. The gel purified insert was then ligated with the retro-virus vector pLNCX2 at different ratios. The products of these ligation reactions were cloned and sequenced to verify the right inserted size and sequence direction. Using this strategy we failed repeatedly to obtain our insert back. Therefore, we changed our cloning strategy into TOPO TA PCR Cloning. First, we added Poly(A) into our PCR product. Then we cloned the mouse AhR PCR product into the pCR2.1-TOPO vector of TOPO TA PCR Cloning kit.

The vector seems to be cut only by XhoI. This proved that there was one enzyme site, *CIa-I* site, was either muted or there was an error in the primer sequence. We redesigned new primers with a different restriction site, ***Sal-I***. and repeated PCR as well as cloning procedure. Fig 2. showed the digestion of pCR2.1-TOPO vector by XhoI and Sal-I, which gave the expected right size for our insert (AhR). We picked some colonies for sequence verification. The correct sequence for the insert was confirmed.

AhR →
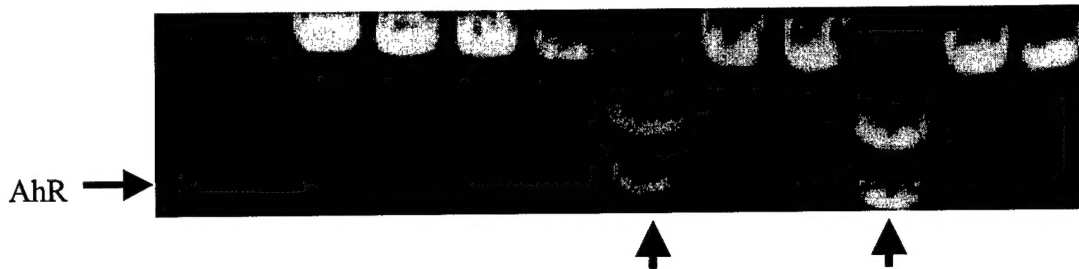
Fig 2. The digestion of pCR2.1-TOPO vector by Xho-I and Sal-I

The next step was to ligate this right insert, which is digested form pCR2.1-TOPO vector into pLXCN2 retroviral vector of the Retro-X-System.

2. Sub-cloning in the Retro-X-System:

At first we used the DH5α competent bacterial cell line, which was recommended by Retro-X system provider (Clontec) instruction manual, to amplify the retro-virus vector pLNCX2. To open the vector, we did the combo restriction enzymes Xho-I and Sal-I digestion. However, when we tried to ligate the AhR insert into these sites we failed. After five times trials with different ratios of the insert and the open vector, we concluded that this retrovector must have mutated when was amplified in the DH5a, which was confirmed when we found out that one of the enzymes, Sal-I, was unable to cut the vector. Fig 3 showed vector form bacterial is unable to be digested by different concentration of Sal-I. This was probably due to this retrovirus vector being instable when amplified at 37°C in this DH5α bacterial cell line.

| ladder | Sal-I
+
Xho-I | Xho-I | high
[Sal-I] | low
[Sal-I] | control
vector
(uncut) |
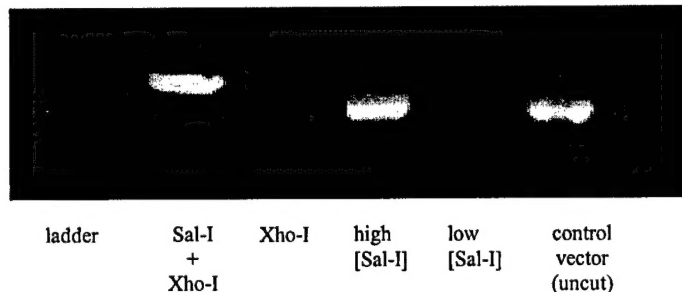
Fig 3.The restriction digest of pLNCX2 vector cloned in DH5α bacteria

We decided to use the retro-vector directly form the original stock from the kit, so we linearized it using double digestion with XhoI and Sal-I and ligated with our mouse AhR insert and cloned it in the XXX10Golden bacterial cell (Biolabs, New England), which was recommended for unstable vector. We successfully obtained colonies, which have the right DNA size. This is shown on Fig.4. Unfortunately, results form sequencing could not verify our insert sequence, which we have identified by restriction digest.

ladder   (digested by two restriction enzymes: Sal-I and Xho-I)   uncut
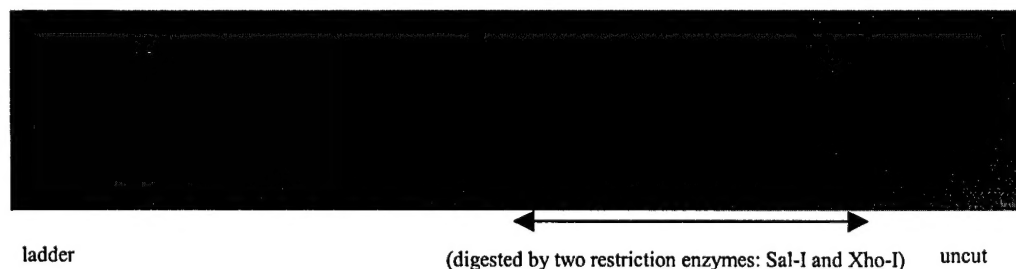
Fig 4. The restriction digest of pLNCX2 vector cloned in XXX10Golden bacterial cell line

After months of these trials and errors with Clontech kit, we decided to stop using this kit and shift to a retroviral vector with Green Fluorescence protein (GFP) gene as a screenable marker. A new vector, pBMN-I-GFP was made available to us as a kind gift from Dr. Gary Nolan, of Stanford University. This vector and its associated reagents also came with very helpful un-limited technical support through their web site

3. Sub-cloning of mouse AhR cDNA into pBMN-I-GFP Vector:

We designed new PCR primers, which contain restriction sites for SacII and XhoI, based on the cloning sites of the pBMN-I-GFP vector. We sub-cloned the mouse AhR cDNA PCR product into pCR2.1-TOPO TA cloning vector and when sequenced it showed that it contains the right sequence. This insert was then digested by SacII and XhoI form TA vector, and ligated into the open pBMN-I-GFP vector. After ligation and cloning in STLB2 bacterial cell line at 30°C we picked multiple positive colonies for restriction digestion with SacII and XhoI, and from the colonies which gave the right size insert we sequenced to verify the right mouse AhR gene sequence.
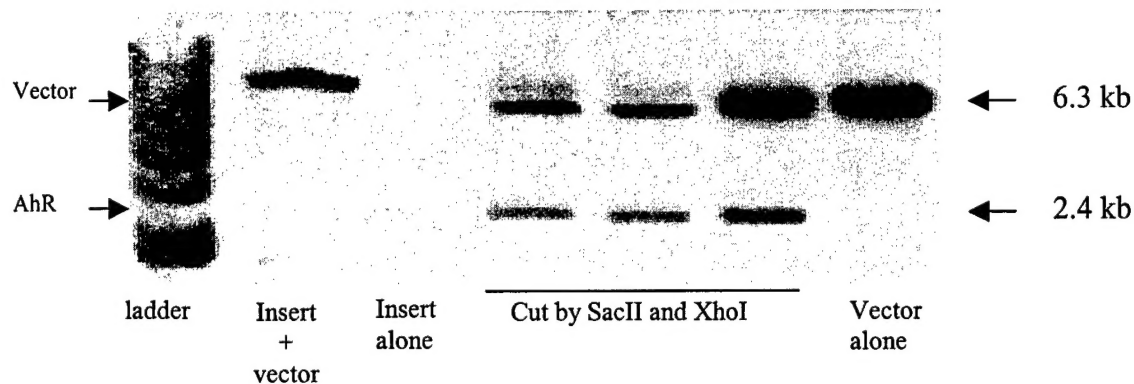

Fig-5 showed the restriction sizes of the pBMN-I-GFP vector and mouse AhR insert

4. Production of high titer concentrated virus stocks and testing for functional expression:

We transfected the pBMN-I-GFP vector control or AhR-containing into the stable packaging cell lines (PT67), and the population of cells expressing the stably integrated retro-vectors were obtained by antibiotic selection. The viruses containing the respective genes were produced from these cells in fresh media and were collected, filtered, aliquoted and stored at −80°C. Before attempting to use these viruses in the mice, we wanted to make sure that they are expressing the GFP and the AhR genes, so we used some of the viral aliquots to infect immortalized human mammary epithelia cell line H16N2. The results were very promising, where we observed both the expression the AhR protein by Western blotting of protein extracts of these cells (Fig 6, below) and the green fluorescence in these cells (Fig. 7 below), five days post the infection.
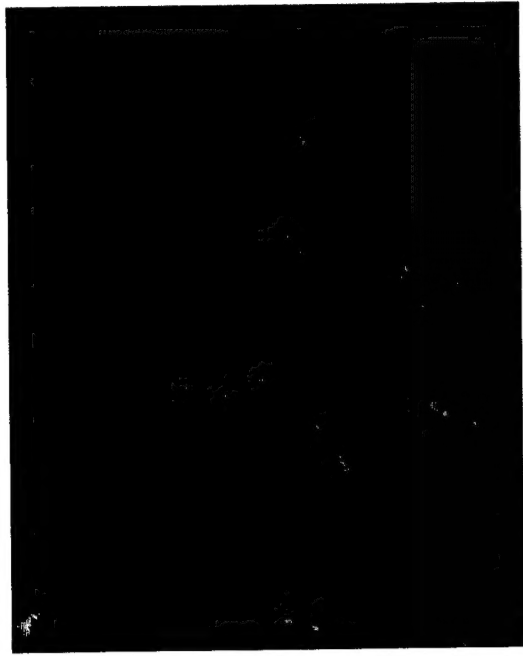
7
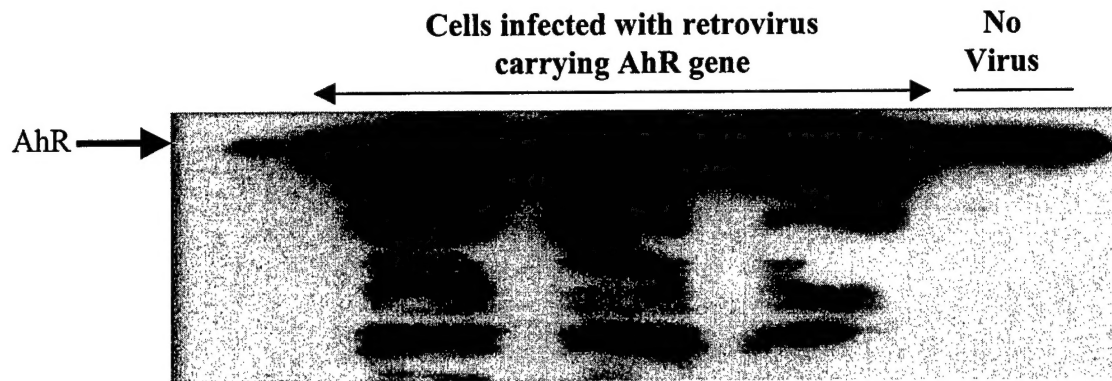
Fig 6. Mouse AhR protein expression in H16N2 Immortalized Human Mammary epithelial Cells, following infection with pBMN-GFP retrovirus vector carrying mouse AhR cDNA.

Control-GFP vector
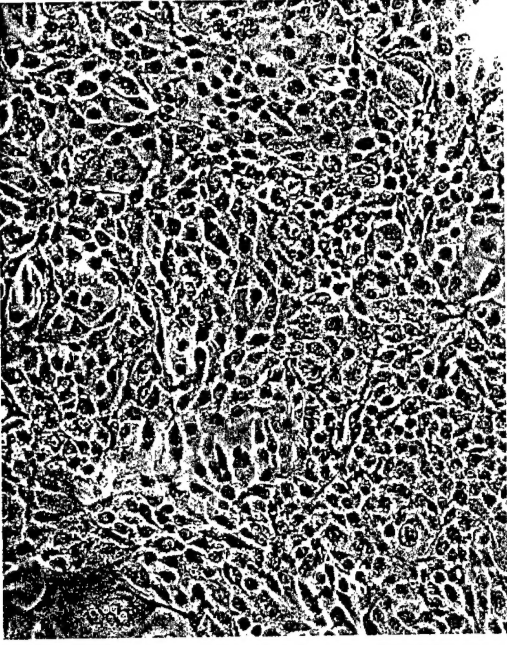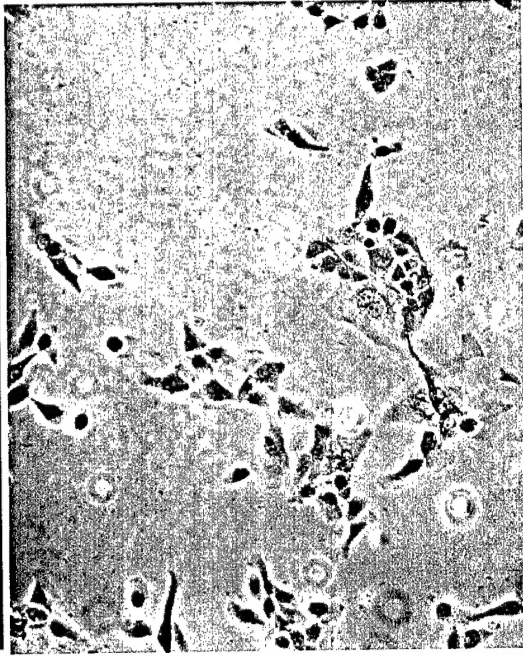
AhR-GFP vector
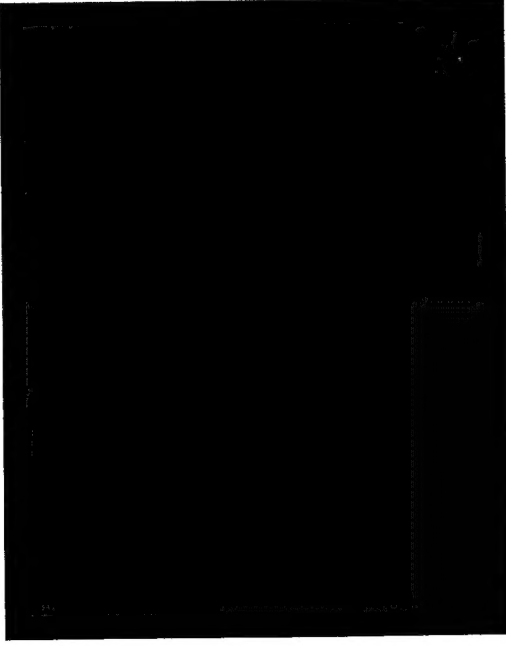
**Fluorescence Image**

Phase contrast

Fig. 7. Expression of GFP retroviral vectors carrying the mouse AhR or control vector, in H16N2 Immortalized Human mammary epithelial cell line, five days post-viral infection

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5. Construction of the recombinant retroviral expression vector for Human AhR:

The full-length human AhR cDNA was amplified by PCR using the proofreading enzyme, *Pfx* and adaptor primers flanked by two restriction enzyme sites, *BglII* and *Cla-I* The amplified product was then purified:

Amplified/
Unpurified
AhR ← ——— Purified AhR ———→

2.5 kb →

Fig9. showsPCR product of Human AhR cDNA
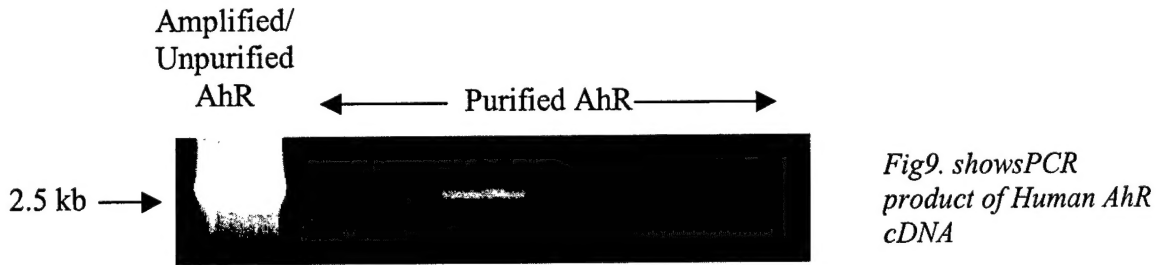
The purified product was then cloned into invitrogen's PCR-Blunt TOPO vector.

← ——— AhR + pCR-Blunt II TOPO vector ———→

6.0 kb →
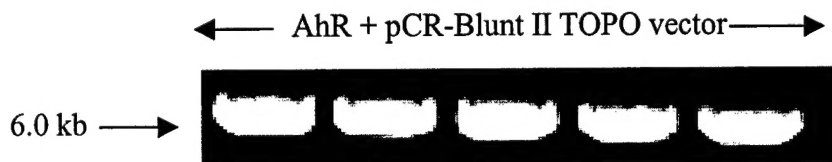
The recombinant vector was then digested using restriction enzymes *Bgl-II* and *Cla-I*, to obtain the AhR insert for subcloning:

TOPO Vector →

AhR →

← —3.5 kb

← —2.5 kb

Fig10.shows pCR-Blunt II-TOPO Vector digestion with Bgl II and Cla-I

Once the correct restriction fragment sizes were obtained, selected clone samples were sequenced. The correct sequence of the insert was verified to be the correct one using MAC vector software.

6. Animal Breeding and establishment of the AhR-Knock-Out mice colony:

We received 5 female heterozygous AhR$^{+/-}$ and 2 homozygous male AhR$^{-/-}$ mice from our collaborator Dr. Bradfield (University of Wisconsin), and they were housed and bred at Meharry Medical College Animal Care. Due to the fragility of the homozygous knockout mice they did not have a good survival rate of litters in the first few weeks so we had to request 10 more pairs of heterozygous animals, and we enforced a higher measures of hygiene and sterile conditions. Right now, we are almost up to our target number of animals. We maintain a very strict genotyping protocol to ensure maintenance of our pure homozygous knockout mice.

7. Pilot studies in mice:

We already have started this week to run our pilot studies to optimize conditions for viral intra-mammary infusion and expression efficiency. Some of the problems we are currently addressing include the volume of injection, and the time consuming process of finding the mammary teats in such young animals. We are

considering an alternative approach if this becomes a major problem; we might surgically clear the mammary glandular tissue and infect it in culture, and then transplant it back in the mammary fat pad of the same donor.

Objective 2: *Blocking of AhR expression in highly metastatic Sager's 21MT human breast carcinoma cell line by stably transfecting antisense human AhR cDNA.*

We originally proposed to block the expression of AhR in metastatic cells using the anti-sense approach, however, in the light of the new advancement in using small interfering RNA (siRNA) technology in silencing gene transcription, we chose to modify our original aim and develop this technique to use it to block the AhR expression in metastatic cells.

1. *Construction of AhR-specific short hairpin (shh) RNAs:*

   a. **Selection of shh RNA sequences**

We selected target regions from the open reading frame of human AhR cDNA sequence. The target regions selected were at least 50nt downstream of the start codon to avoid possible sites of regulatory protein binding. A search was then done to select sequences of 5'AA(N19)UU with a 32-79% G/C content. The shhRNA sequences chosen by this criteria were blast-searched against human EST libraries to ensure that only a single gene is targeted.

   b. **Preparation of shh RNA duplexes**

Four nucleotide sequences fitting these criteria were identified. Four corresponding oligonucleotides were then designed which contained the corresponding AhR sequence, plus a six nucleotides spacer that does not correspond to any AhR sequence and the complementary and anti-parallel sequence.
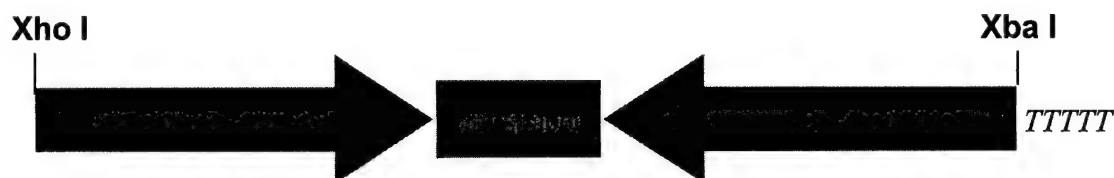
Each of the four oligonucleotides was also designed with an XhoI restriction site at the 5' end and an XbaI restriction site at the 3' end. The restriction sites were designed to ensure the correct restriction fragment is cloned into a vector which is already digested with XbaI and SalI(compatible with Xho I). Each oligonucleotide is designed along with an oligonucleotide of complementary sequence. The two complementary oligonucleotides were annealed prior to ligation into the pSHH vector. Once ligated into the vector, the sequences of the inserts were verified using a primer set designed for the pSHH vector. The pSHH vector carrying each construct also carries neomycin resistant gene as a selective marker for mammalian cells. We have successfully synthesized, cloned and verified the sequences of these four transcription silencers. We are in the process of transfecting them singly and in cocktail to permenantly integrate them into our experimental cells (the metastatic MT-2 human breast carcinoma cell lines which over-express the AhR).
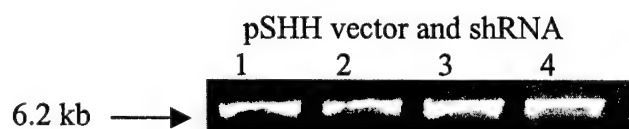
Objective 3: *To determine the status of the AhR activation in the Sager's cells in presence or absence of TCDD treatment.*

We examined the expression and the activation status of the AhR in the Sager's cells in presence and absence of the receptor agonist, Dioxin. In appendix 1, panel A is a Western blot of the Ah receptor level in control (C) cells and in cells treated with Dioxin (T) for 20 hours. It is well established in the AhR field that treatment with dioxin will result in the nuclear translocation of the Ah receptor from its normal cytosolic localization. Such nuclear localization is usually accompanied by a dramatic down-regulation of the receptor protein with hours, and it gets depleted to more than 90% by 24h treatment. Our data indicated such behavior of the receptor depletion only in the normal immortalized mammary epithelial cell line and the early tumor cell lines (PT &NT), while the MT-2 metastatic cell line were resistant to the effect (Panel A). Immunochemical staining and fluorescence localization of AhR protein in these cells has confirmed these observations and also clearly demonstrated the presence of constitutive activated Ah receptors in the advanced carcinoma cell lines (Sager's cells), as evidenced by their nuclear localization in absence of ligand treatment (control). Interestingly, in the primary culture of normal human mammary epithelia cells, the Ah receptor was predominantly cytosolic (Appendix 2). The cell fractionation experiments confirmed the data from immunofluorescence experiments (Appendix 1, Panel C).

The ligand-activated nuclear localization of AhR is usually associated with its role in enhancing the transcription of its regulated genes. Therefore, we examined the expression of two of these genes (CYP1A1 and CYP1B1). However, while treatment with dioxin for 22h has resulted in a substantial induction of the protein products of these genes, there was no detectable expression of CYP1A1 and there was very low level of CYP1B1 in absence of ligand (Appendix 1, Panel D). Therefore, its obvious that the nuclear localization of AhR in these advanced breast cancer cell lines is not related to its function in ligand-activated transcription of genes involved in mediationg toxic response of chemicals.

We have also examined the interaction of the AhR with cytoskeleton proteins, and we observed a remarkable association between AhR and cyto-keratin 18 (CK-18) in both primary culture of normal human mammary epithelia cells (HMECs) and breast carcinoma cell lines (HBC). While there was a co-localization of CK-18 and AhR in the untreated HMECs, but not in the dioxin-treated ones, the co-localization of AhR and cK-18 in the metastatic Sager's HBC was both under no treatment and when dioxin-exposed (Appendix 3).

We are in the process of compiling data on this aspect of the AhR in these cells to be submitted for publication.

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Key Research Accomplishments

1. The construction of the recombinant retroviral expression vector for mouse AhR, and the production of the retro-viruses carrying this gene.

2. Demonstrated that these viruses are capable of stably expressing both GFP and AhR in cultured cells and animal tissues at very high levels.

3. The construction of the recombinant retroviral expression vector for human AhR.

4. Design and synthesis of four siRNA for human AhR, and ongoing testinf of their functions.

5. The demonstration of the constitutive activation of AhR in advanced breast carcinoma cell lines, and its association with the cytokeratin 18

Reportable Outcomes

- I have a graduate student (Ms Joann Brooks), who is training under my guidance on breast cancer pharmacology. She is working on the human cell line portion of this project. She has applied and obtained a Pre-doctoral Traineeship Award from the Department of Defense- Breast Cancer Research Program (DOD-BCRP).
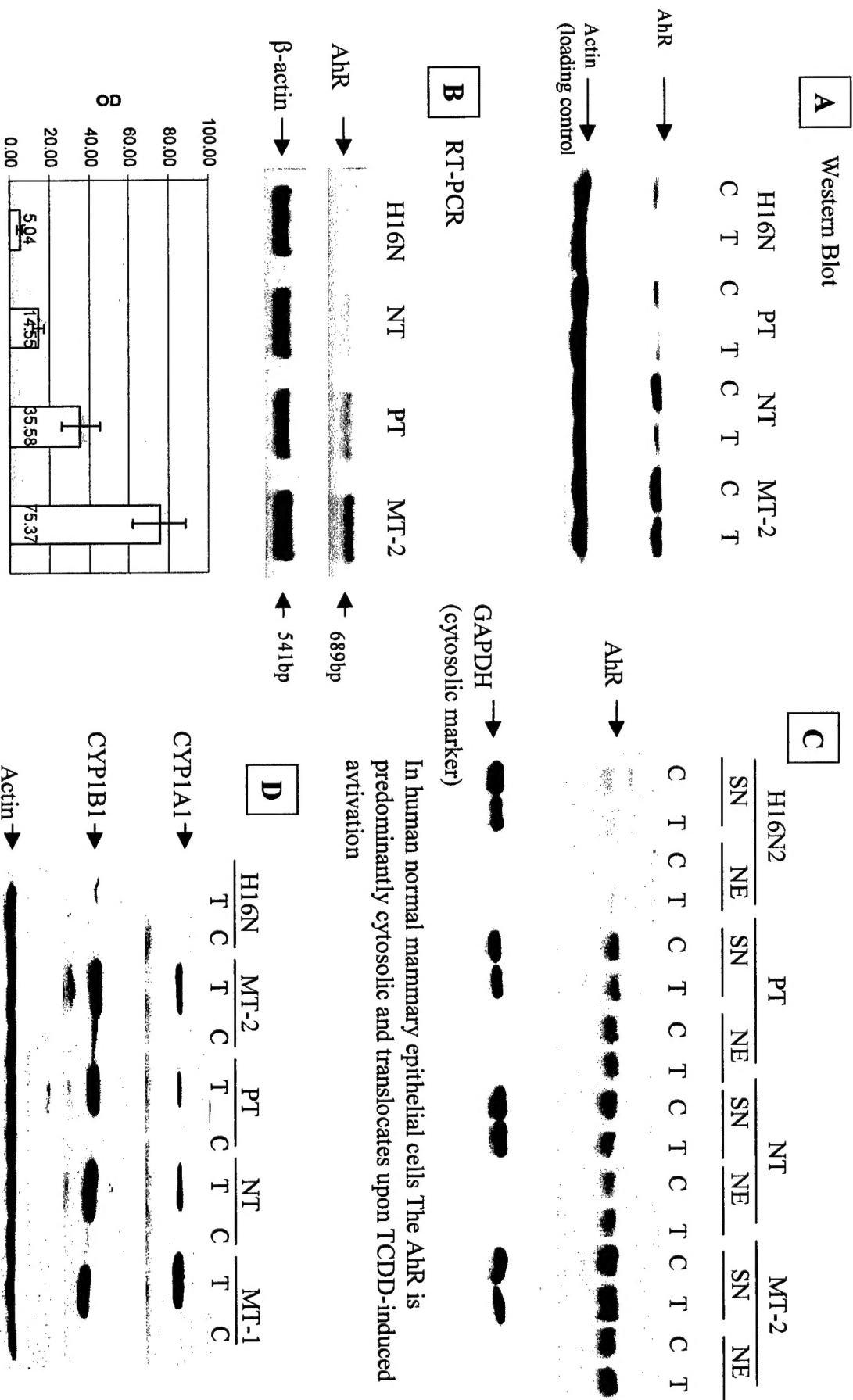
Conclusions

I think we are making a very substantial progress, and in this past year, we have overcome most of our anticipated technical difficulties. Therefore, we expect this coming year to be a very productive year, where we will be able to harvest data related to our proposed studies. Even though the data we presented in this report are not confidential in the sense, however, we ask for consideration of our vulnerable situation since we have not published any of these data, including the development of theses valuable reagents.

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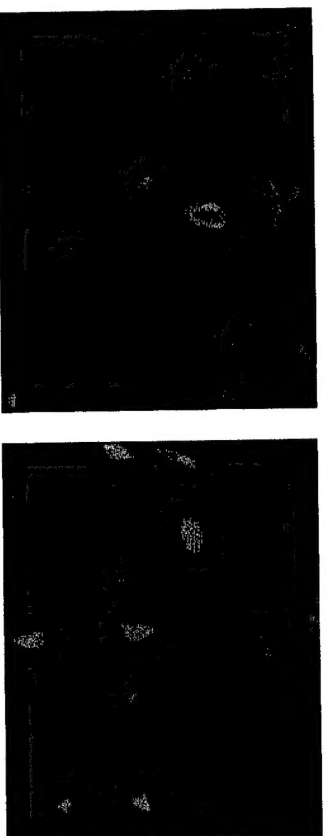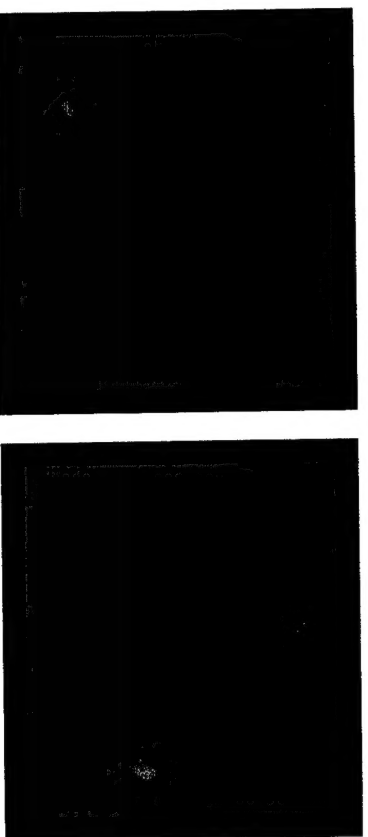
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A Western Blot

H16N   PT   NT   MT-2

Actin
(loading control)   C T C T C T C T

AhR

B RT-PCR

H16N   NT   PT   MT-2

β-actin ➝   ⬅ 541bp

AhR ➝   ⬅ 689bp

OD

100.00
80.00
60.00
40.00
20.00
0.00

5.04
14.55
35.58
75.37

Elevated levels of the AhR in the advanced malignant
HBC cell lines: Both at protein and mRNA levels

C

H16N2   PT   NT   MT-1

SN NE | SN NE | SN NE | SN NE

C T | C T | C T | C T | C T | C T | C T | C T

GAPDH ➝
(cytosolic marker)

AhR ➝

In human normal mammary epithelial cells The AhR is
predominantly cytosolic and translocates upon TCDD-induced
avtivation

D

H16N   MT-2   PT   NT   MT-2

T C | T C | T C | T C | T C

CYP1A1 ➝

CYP1B1 ➝

Actin ➝

Expression of TCDD-induced CYPs in Sager's Cell Lines

Appendix 2

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Primary Human Mammary Epithelial Cells

AhR in H16N2 Immortalized normal mammary epithelial Cells

Sager's 21NT Breast carcinoma Cell lines
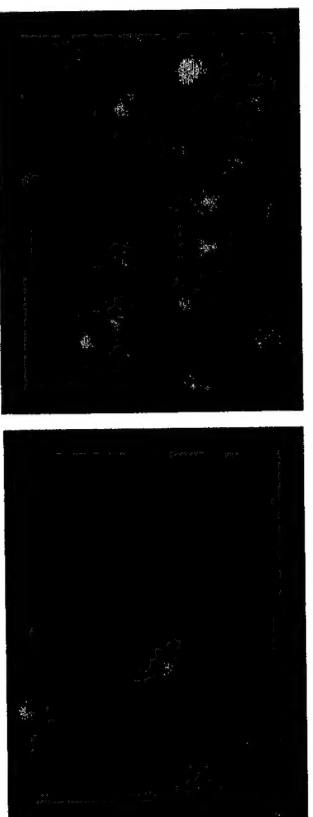
Sager's 21MT-2 Breast carcinoma Cell lines

The Metastatic Sager's cell line 21MT-1
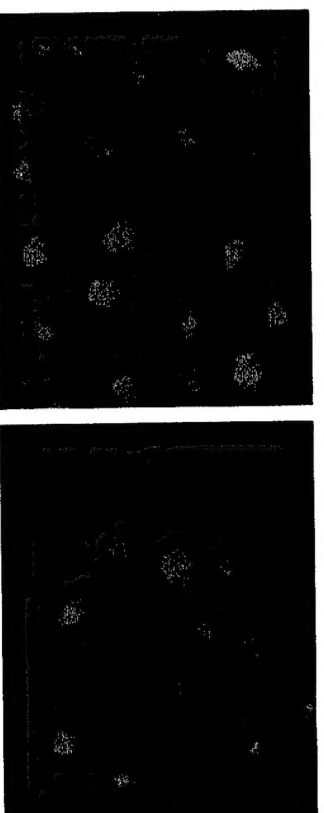
Control

TCDD

Control

TCDD

In human normal mammary epithelial cells The AhR is predominantly cytosolic and translocates upon TCDD-induced avtivation

The Ah receptor in the advanced breast cancer cell lines is constitutively activated as evidenced by its nuclear localization in absence of ligands
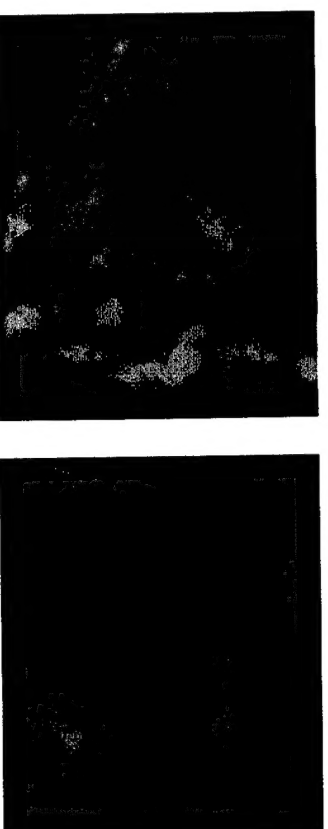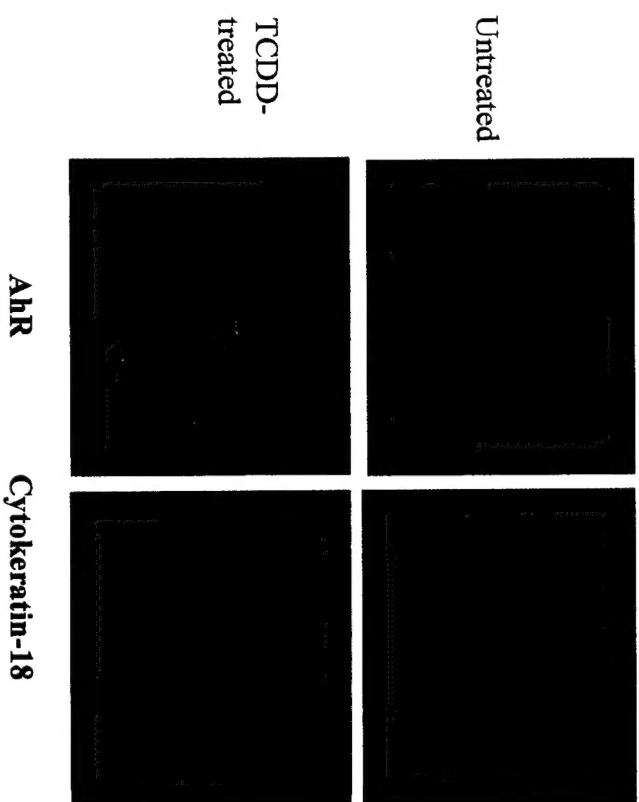
PI: Eltom, Sakina E, DVM, PHD

Human Primary Mammary Epithelial Cell culture

The Metastatic Sager's cell line 21MT-1- TCDD

Untreated

TCDD-
treated

AhR   **Cytokeratin-18**

Un-
treated

TCDD-
treated

TCDD-
treated
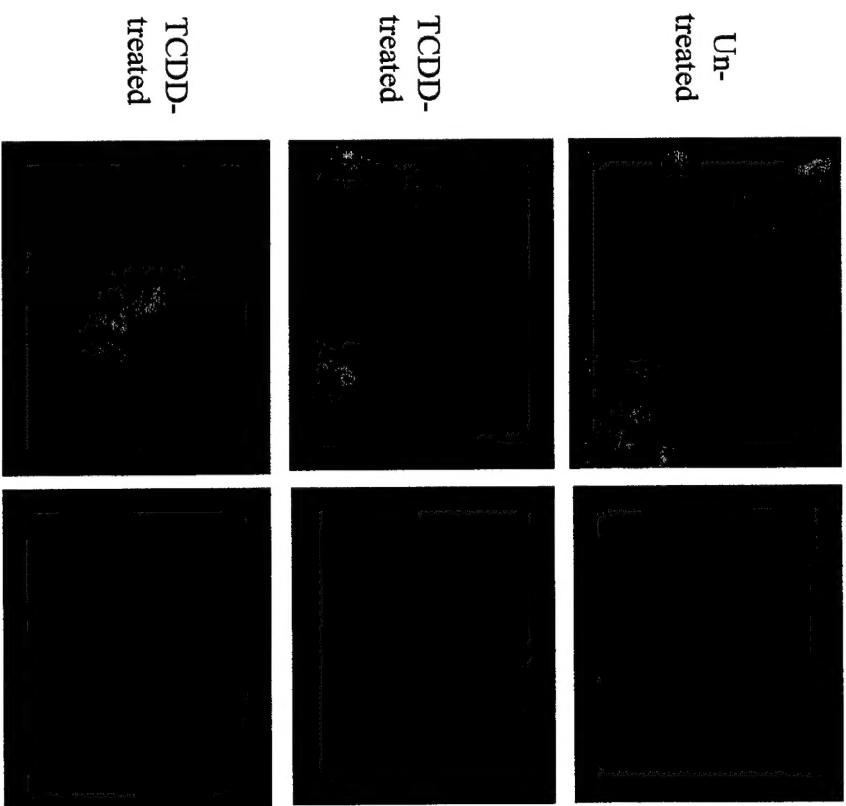
AhR   **Cytokeratin-18**

There is a co-localization of cytokeratin-18 and the AhR in the untreated primary mammary epithelial cells but not the TCDD-exposed ones.
Co-localization of AhR and cytokeratin-18 in the metastatic cell line 21MT-1, both under no treatment and when TCDD- exposed.